active site inhibitors to these enzymes the highly charged nature of the substrate binding site has yielded relatively intractable chemotypes for drug discovery. We have discovered a novel allosteric site in both apoptotic capsases −3, and −7 and the inflammatory caspase−1. This involved the use of a site-directed fragment-based approach to drug discovery, called Tethering® or disulfide-trapping. In this case a native thiol at the dimer interface of caspases was allowed to react reversibly under thiol exchange conditions with a small library of disulfide-containing small molecules. The site-directed character of the approach can focus the discovery process on unactivated enzymes and allosteric sites which would otherwise be difficult to selectively target. We identified selective tethered compounds for these allosteric sites and will present their structures and mechanisms for inhibition (Hardy et al. PNAS *101*, 12461 (2004); Scheer et al., PNAS *103*,7579 (2006)). Mutational analysis reveals an "allosteric circuit" that connects these sites and we believe is naturally involved in their regulation. We have further developed chemical and immunologic tool that trap these transitions so they may be studied in vitro and in cells.

Platform AX: Membrane Proteins - III

2600-Plat Lateral Sorting of Influenza Virus Hemagglutinin in Membranes

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Hemagglutinin (HA) is a homotrimeric glycoprotein embedded in the envelope of Influenza virus. It mediates binding of the virus to the host cell as well as fusion between the viral envelope and the endosomal membrane. HA has been reported to play also an important role in budding of the new viral particles from the host cell. Rafts reflecting liquid-ordered lipid domains enriched of sphingomyelin and cholesterol have been suggested as sites for local recruitment of viral components. HA is supposed to entrap in those lipid domains. In order to elucidate the role of the HA transmembrane domain in lipid raft localization we expressed constructs harboring the transmembrane domain and the cytoplasmic tail but lacking the N-terminal ectodomain of HA in the plasma membrane of mammalian cells (MDCKII, CHO-K1). The N-terminus of the transmembrane domain was tagged with YFP (HA-YFP). We studied Foerster's energy transfer (FRET) between the artificial HA-YFP and a GPI anchored CFP as a raft marker by fluorescence lifetime imaging microscopy (FLIM). First results suggest that HA constructs are indeed sorted and enriched into cholesterol dependent lipid domains indicated by enhanced FRET efficiency. This is supported by the observation that cholesterol depletion of the plasma membrane caused a significant decrease of FRET. Likewise, deletion of the three highly conserved palmitoylation sites of HA is also accompanied by a reduction of FRET efficiency. Taken together, the results are in agreement with sorting of HA constructs into cholesterol-enriched lipid domains.

2601-Plat Solution Structure of Integral Membrane Protein from *Mycobacterium tuberculosis*: Insight into Rv0008c - an FtsZ Inhibitor and Its Complex

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Our goals are to determine the structure of Rv0008c, an FtsZ inhibitor, a critical component of the cell division machinery from *Mycobacterium tuberculosis* and to study the binding and interface of this protein and Rv0011c by solution NMR and biophysical methods. Rv0008c is a small, conserved integral membrane protein consisting of 145 residues. It is in a gene cluster that we have shown to form a multi-protein complex with Rv0010c and Rv0011c - a homologue of CrgA, an FtsZ inhibitor. Rv0008c is a potential tuberculosis drug target. A detailed three-dimensional structure of this protein will help to understand its critical role in FtsZ depolymerization and therefore this will help to suggest a possible cell division control mechanism in *M. tuberculosis*.

Backbone assignments of Rv0008c were obtained from 2-D and 3-D TROSY-based experiments on uniformly $^{13}C/^{15}N$-labeled Rv0008c protein in DPC micelles. Residual dipolar couplings were obtained for protein in compressed polyacrylamide gels by using gNtrosyS3 experiments. Paramagnetic relaxation enhancement (PRE) distances have been measured by using site-directed parallel spin-labeling method. These distances were derived by comparing the difference in peak volumes in the presence of paramagnetic or diamagnetic agents based on the HSQC experiments. We have completed 90% of the backbone assignments and achieved two different alignments for Rv0008c in uncharged and positive charged acrylamide gels.

We have obtained a good number of PRE distances for nine spin label sites and have calculated the initial structure for Rv0008c. We have also identified and mapped out the residues involving in the interaction of Rv0008c and Rv0011c. In addition, mutations have been made in the transmembrane helices of Rv0008c and Rv0011c to further study the interaction of Rv0008c and Rv0011c.

2602-Plat Controlling the Inhibition of the Sarcoplasmic CA^{2+}-ATPase by Tuning Phospholamban Structural Dynamics

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Cardiac contraction and relaxation are regulated by conformational transitions of protein complexes that are responsible for calcium trafficking through cell membranes. Central to the muscle relaxation phase is a dynamic membrane protein complex formed by Ca^{2+}-ATPase (SERCA) and phospholamban (PLN), which in humans is responsible for approximately 70% of the calcium re-uptake in the sarcoplasmic reticulum. Dysfunction in this regulatory mechanism causes severe pathophysiologies. In this paper, we use a combination of nuclear magnetic resonance, electron paramagnetic resonance, and coupled enzyme assays to investigate how single mutations at position 21 affect PLN structural dynamics and, in turn, its interaction with SERCA. We found that it is possible to control SERCA activity by tuning PLN structural dynamics. Both increased rigidity and mobility of the PLN backbone cause a reduction of SERCA inhibition, affecting calcium transport. While the more rigid, loss-of-function (LOF) mutants have lower binding affinities for SERCA, the more dynamic LOF mutants have binding affinities similar to that of PLN. Here, we demonstrate that it is possible to harness this knowledge to design new LOF mutants with activity similar to S16E (a mutant already used in gene therapy) for their possible application in retro-adenovirus gene therapy. As proof of concept, we show a new mutant of PLN with improved LOF characteristics *in vitro*.

NIH Grants GM64742 and K02HL080081 to GV.

2603-Plat Cardiac Calcium Transport Regulation Probed by DEER

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We are using solid-phase peptide synthesis (SPPS), site-directed spin labeling, and pulsed EPR to investigate the structural dynamics and protein interactions of cardiac calcium regulation. In the heart, SERCA activity is modulated by phospholamban (PLB), a 52-residue integral membrane protein that forms a homopentamer but binds to SERCA as a monomer. Fluorescence (Mueller et al, 2004) and EPR (Karim et al, 2006) data from our lab have shown that

(a) monomeric PLB binds to SERCA with high affinity and inhibits activity and
(b) increasing calcium concentration to micromolar levels or phosphorylating PLB at Ser16 relieves inhibition but does not dissociate PLB from SERCA.

DEER is a pulsed EPR technique capable of measuring distances up to 6 nm between spin labels attached to proteins. We have used DEER to measure distances within the PLB pentamer. In conjunction with NMR measurements on this system (Traaseth et al., PNAS, in press), these long-range distance constraints are being used to solve the structure of the pentamer ± phosphorylation. We have spin labeled SERCA at Cys674 in the P-domain and are measuring SERCA self-association as affected by PLB (inhibitory) and PLB phosphorylation (activating). We are also measuring distances between selected sites on SERCA and PLB, to determine the structure of the SERCA-PLB complex in both inhibitory and activating states.

This work was supported by NIH (GM27906, RR22362), AHA (0615710Z), and the Minnesota Supercomputing Institute.

2604-Plat EPR and Solid-State NMR Studies of Membrane Proteins

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Magnetically aligned (bicelles) and mechanically aligned (glass-plate) phospholipid bilayers have been successfully used in a wide range of solid state NMR (SSNMR) and solution NMR studies to macroscopically order both membrane bound and water soluble macromolecules. Sample orientation enables the efficient high-resolution measurement of anisotropic spectral parameters that provide valuable structural and dynamic information for both EPR and NMR spectroscopic studies. In particular, several researchers have investigated membrane proteins and peptides incorporated into mechanically aligned phospholipids bilayers with solid-state NMR spectroscopy. However, for the first time we demonstrates the feasibility of obtaining topology and helical tilt information of an integral membrane protein inserted into various alignment media such as bicelles, nanomembranes, and glass plates using a spin label EPR approach. A rigid nitroxide spin label attached to a helix of a protein was used to elucidate the structural topology and the tilt of the helix with respect to the membrane normal through the measurement of orientational dependent hyperfine splitting values. The advantages and disadvantages of using various alignment approaches will be discussed. EPR spectroscopy is approximately a 1000 fold more sensitive than solid-state NMR spectroscopy. Thus, the helical tilt of an integral membrane peptide can be determined much more efficiently when compared to NMR techniques.

2605-Plat Backbone Conformation and Dynamics of the Lipid-Modified Membrane Anchor of Human N-Ras Investigated by Solid-State NMR and Molecular Dynamics Simulations

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Many proteins involved in signal transduction are anchored to membranes by covalently attached lipid modifications. In this study we investigated the conformation and dynamics of the backbone and side chains of the N-Ras membrane anchoring domain. Experimental solid-state NMR studies involved doubly lipid-modified uniformly ^{13}C and ^{15}N labeled heptapeptides representing the C-terminus of N-Ras that were incorporated into DMPC bilayers. A structural model of the peptide was calculated on the basis of isotropic chemical shifts, explicit torsion angle measurements, and nuclear Overhauser effects determined by solid-state NMR. The amplitude of molecular motions was assessed by ^{1}H-^{13}C order parameter measurements using separated local field NMR. For the determination of the correlation times of motion, T_1 and T_2 relaxation times were measured and analyzed using a modified Lipari-Szabo approach. To further understand the dynamics of Ras, a molecular dynamics simulation of the molecule in a lipid bilayer

was conducted. In generating starting conditions for the simulation special attention was paid to the backbone conformation since transitions between conformations were found to be rare events in a previous simulation of 100 ns length on this system [1]. Therefore, the experimentally determined conformation of the peptide backbone was equilibrated using a replica exchange technique, in an explicit membrane environment, to identify different confomers and their relative probability. Subsequently, the resulting distribution of conformations was used for a long conventional MD simulation that was analyzed with regard to the experimental data. Through such a combined approach a detailed model of the dynamics of the peptide was obtained.

References

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2606-Plat PHLIP - pH (low) Insertion Peptide: Biophysical Studies and Medical Applications

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pHLIP (pH (Low) Insertion Peptide) is a membrane peptide with three states:

(I) soluble in a random configuration in water near neutral pH,
(II) bound in a random configuration to the surface of a membrane near neutral pH, and
(III) inserted across the membrane as an α-helix at acidic pH.

At low concentrations, pHLIP is a monomer in all three states. pHLIP insertion into lipid bilayers, human red blood cells and cancer cells *in vitro* and *in vivo* occurs as a result of protonation of Asp residues due to a decrease of pH. Protonation enhances peptide hydrophobicity and increases pHLIP affinity for a lipid bilayer 20 fold in comparison with neutral pH. The peptide does not induce fusion or membrane leakage. With low pH driving the process, pHLIP can translocate cargo molecules attached to its C-terminus via a disulfide and release them in the reducing environment of a cell cytoplasm. Among translocated molecules are fluorescent dyes, a hydrophilic cyclic peptide (phalloidin) and gene regulation agents (PNA, peptide nucleic acids). We have shown the ability of pHLIP to specifically target acidic tissue *in vivo* and to induce protein expression by PNA. The unique properties of pHLIP made it attractive for the biophysical investigation of membrane protein folding *in vitro* and for the development of a novel class of delivery peptides for the transport of therapeutic and diagnostic agents to acidic tissue sites associated with various pathological processes *in vivo*.

This work was supported in part by grants from the DoD PCRP CDMRP PC050351 and Champlins Foundation to YKR; from the DoD BCRP CDMRP BC061356 and NIH NCI 1R21CA125280-01A1 to OAA, and from the NIH GM073857 and GM070895 to DME.

2607-Plat Affinity of a Sphingolipid Binding Domain Peptide to Glycosphingolipid Receptors

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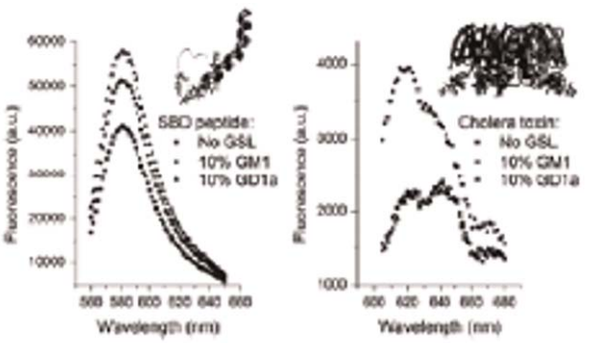
A fluorescently labeled peptide, the sphingolipid binding domain (SBD), is created as a tool to trace sphingolipid behavior and trafficking in neurodegenerative models. SBD, which consists of a 25 amino acid fragment of the Alzheimer's disease associated Aβ peptide, is TAMRA-coupled and applied to liposomes containing a raft-like mixture of sphingomyelin (SM), cholesterol (Chol), palmitoyl-oleoyl-phosphatidylcholine (POPC), and spiked with different glycosphingolipids. Such lipid compositions allow us to mimic a typical cell membrane with lipid microdomains. To assess the affinity of these different mixtures for SBD, unbound SBD are separated from liposomes by centrifugation. Fluorescence spectroscopy reveals a higher affinity of SBD for GD1a than for GM1; for comparison, fluorescently labeled cholera toxin binds as expected much more strongly to its receptor GM1 (Figure). A very poor affinity is found for control POPC-only liposomes, demonstrating the requirement of sphingolipid and cholesterol, the components of lipid microdomains, for binding. These peptide-bound microdomains will be investigated further by atomic force microscopy.

Figure. Fluorescence spectroscopy of liposomes with bound fluorescence-tagged SBD peptide (left) and cholera toxin (right). POPC/SM/Chol (45/25/30) liposomes were made with or without 10% in weight of glycosphingolipid receptors GM1 or GD1a.



Platform AY: Single Molecule Biophysics - III

2608-Plat Insights into the Nucleation of SH3 Amyloid Fibril Formation Using Single Molecule Fluorescence

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The tissue deposition of the [beta]-sheet rich, filamentous protein aggregates, amyloid fibrils, represents the common pathological hallmark of a range of degenerative disorders including Alzheimer's and Parkinson's diseases. However, the observation that many